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Identification of intragenic mutations in the *Hansenula polymorpha* *PEX6* gene that affect peroxisome biogenesis and methylotrophic growth

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Abstract

Two interacting AAA ATPases, Pex1p and Pex6p, are indispensable for peroxisome biogenesis in different organisms. Mutations affecting corresponding genes are the most common cause of the peroxisome biogenesis disorders in humans. By UV mutagenesis of the *Hansenula polymorpha* *pex6* mutant, deficient in peroxisome biogenesis, we isolated a conditional cold-sensitive strain with restored ability to grow in methanol medium at 37°C but not at 28°C. Sequencing of the *pex6* allele revealed a point mutation in the first AAA module of the *PEX6* gene that leads to substitution of a conserved amino acid residue (G737E). An additional intragenic mutation identified in the cold-sensitive *pex6* allele leads to a conserved amino acid substitution in the second AAA domain (R1000G). Electron microscopic analysis revealed restored peroxisomes in methanol-induced cold-sensitive *pex6* cells at both permissive and restrictive temperatures. If separated, the secondary mutation did not affect methylotrophic growth. Our data suggest that *H. polymorpha* Pex6p may have a complex function in peroxisome biogenesis in which identified amino acid residues are involved.

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Keywords: Peroxisome biogenesis; *PEX6*; Methylotrophic yeast; *Hansenula polymorpha*

1. Introduction

Accumulated data suggest that mechanisms of peroxisome biogenesis are well conserved among different eukaryotes (reviewed in [1,2]). Yeasts have proved to be a convenient model system to study molecular mechanisms of peroxisome biogenesis. In yeasts, peroxisomes are dispensable for growth unless particular carbon sources, i.e. oleate, methanol, are utilized. Therefore, mutants deficient in peroxisome biogenesis could be efficiently isolated and studied in different yeast species [1–4]. Methylotrophic yeasts have a particular advantage in studying mechanisms of peroxisome biogenesis because of the massive peroxi-

some proliferation they exhibit upon induction by methanol [5].

Among genes involved in peroxisome biogenesis (PEX genes, their products designated peroxins) originally discovered in yeasts are *PEX1* and *PEX6* that encode ATPases belonging to an AAA family [1,6,7]. It was demonstrated that mutations in corresponding human homologues are the most common cause of inherited peroxisome biogenesis disorders (PBD) [8]. The function of the peroxins involved has not been completely elucidated. In yeasts and higher eukaryotes Pex1p and Pex6p physically and functionally interact in vivo, and this interaction is ATP-dependent [8–10]. Based on the model of the yeast *Yarrowia lipolytica*, it was proposed that the Pex1p/Pex6p complex participates in the fusion events of preperoxisomal vesicles [11]. Other data suggest that this complex is required for terminal steps of matrix protein import [12].

In the methylotrophic yeast *Hansenula polymorpha*, de-

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iciency in the *PEX6* gene impairs peroxisome matrix protein import: upon methanol induction membranous peroxisome ‘ghosts’ are present in the cells and peroxisomal enzymes, such as alcohol oxidase and catalase, are mislocalized to the cytosol [9]. As a result, *pex6* mutants are unable to utilize methanol as carbon and energy source. In this report, we describe a conditional cold-sensitive (cs) *pex6* mutant strain isolated upon introduction of an additional intragenic mutation in the *PEX6* gene of a *pex6* mutant, and identification of the corresponding mutations. Preliminary data on its isolation have been published [13].

2. Materials and methods

2.1. Yeast strains and media

The *H. polymorpha* strains used were: the wild-type strains CBS4732 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), NCYC495 *leu1-1* (National Collection of Yeast Cultures, Food Research Institute, Norwich, UK); *pex6 leu1-1* mutant, originally designated *per5-C79* [14], and $\Delta pex6$ strain [9].

Yeasts were grown at 28°C or 37°C in YPD medium (1% yeast extract, 1% peptone and 1% glucose) or selective mineral media (MM) with 0.05% yeast extract [15], supplemented with 1% glucose, 0.5% methanol, 1% ethanol or 0.5% glycerol and 0.5% ammonium sulfate as a nitrogen source. Leucine (40 mg l⁻¹) was added to all mineral media. Yeast cell density was determined by absorbance at 590 nm. For solid media agar (2%) was added. Sporulation/mating media and techniques for *H. polymorpha* were essentially as described [16].

2.2. Molecular techniques

Procedures and conditions used for isolation of plasmid DNA, digestion with restriction enzymes, isolation of DNA fragments, and agarose gel electrophoresis were carried out according to [17]. Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA) and used as indicated by the supplier. The *Escherichia coli* strain DH5 α was used for plasmid propagation.

Electrotransformation of *H. polymorpha* was performed as described [18]. To isolate genomic DNA fragments with mutated *PEX6* genes, total DNAs were isolated from *pex6* and cs *pex6* mutants as described [19]. They served as templates in polymerase chain reactions (PCR) with the primer PEX6-F: 5'-GCTGTCGTCACAATAATG-3', specific for the *PEX6* gene promoter region, and the primer PEX6-R: 5'-ATTACCGGAAGTCGCTTG-3', specific for the sequence downstream of the *PEX6* gene. PCR was carried out with Taq-DNA Polymerase (Promega, Madison, WI, USA) according to the supplier's instructions. Nucleotide sequencing of the isolated PCR products was carried out as described [9]. To exclude potential PCR

amplification mistakes, two independently amplified fragments for each strain were sequenced in both directions.

Sequence alignments were performed using the MultAlign software [20]. ORF Finder, a graphical analysis tool of the National Center for Biotechnology Information (Bethesda, MD, USA) was used for analysis of DNA sequences. Protein sequence homology search was done using the WWW BLAST server.

2.3. Conditional mutant isolation

For mutant isolation, cells of the initial *pex6* strain were grown in liquid YPD, washed twice with distilled water, spread on the MM plates with 0.5% methanol (5×10^7 cells per plate), and UV-mutagenized for 60 s. Plates were incubated for 4–5 days at 28°C or 37°C and growing colonies were then replica-plated on the same medium and incubated at different temperatures to identify conditional mutants.

2.4. Electron microscopic methods

Cells were fixed and prepared for electron microscopy as in [15].

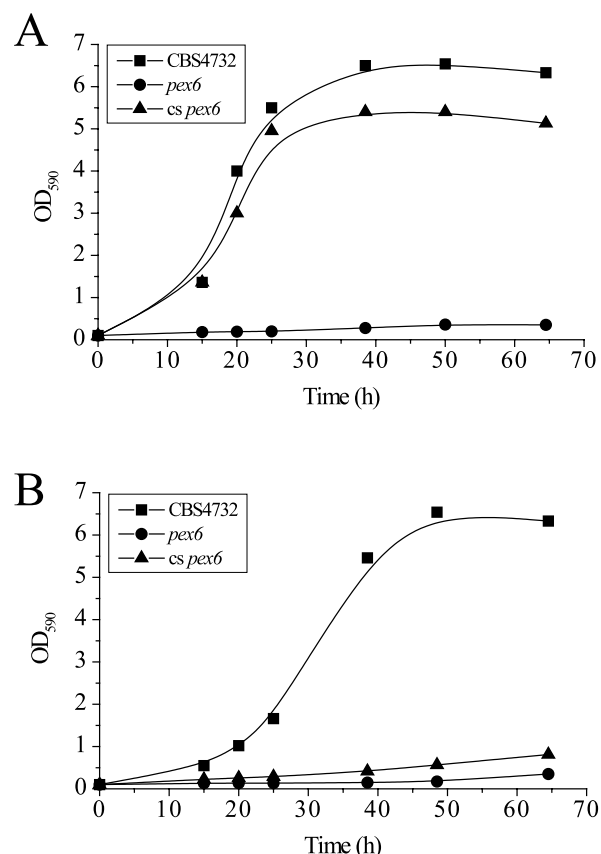


Fig. 1. Growth kinetics of *H. polymorpha* wild-type strain, *pex6* and cs *pex6* mutants in methanol medium at (A) 37°C and (B) 28°C.

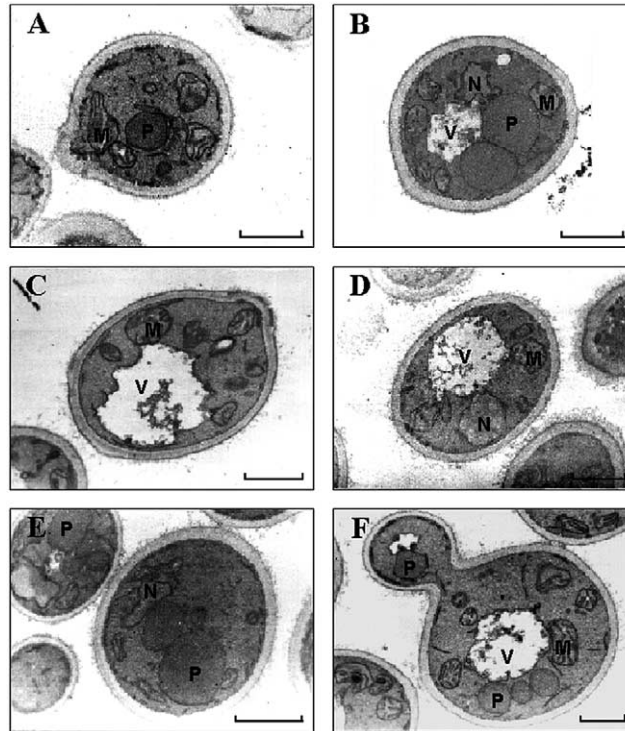


Fig. 2. Morphology of *H. polymorpha* wild-type strain (A,B), *pex6* (C,D) and derivative *cs pex6* (E,F) mutants incubated in methanol MM for 12 h at 28°C (A,C,E) and 37°C (B,D,F). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 1 μ m.

3. Results

3.1. Isolation of the *cs pex6* mutant

We initiated experiments aimed at isolating UV-induced, conditional (ts or cs) revertants from an *H. polymorpha pex6* (*per5-C79*) mutant as an initial strain. The *pex6* cells were mutagenized, and colonies with restored ability to grow on MM methanol plates were selected. It appeared that stable revertants can be isolated from *per5-C79* at a high frequency of 1×10^{-6} . Out of several hundred isolated UV-induced revertants, one conditional (cs) clone with restored growth on methanol at 37°C (permissive temperature) and very slow growth at 28°C (restrictive temperature) was identified (Fig. 1). This *cs pex6* mutant exhibited the cold-sensitive phenotype only in methanol medium, but not in glucose, ethanol or glycerol media (not shown). Therefore, presumably a secondary mutation causative for the conditional phenotype interfered only with methylotrophic growth.

3.2. Ultrastructural characterization of the *cs pex6* strain

To elucidate whether the restored ability to utilize methanol in the *cs pex6* mutant is accompanied by restored peroxisome biogenesis, electron microscopic analysis was carried out. It revealed morphologically detectable peroxisomes at both permissive and restrictive temperatures upon methanol induction (Fig. 2). Peroxisomes with the

wild-type morphology were induced in *cs pex6* cells at the permissive temperature, 37°C (Fig. 2F). No significant difference was observed for the mutant relative to the wild-type strain with regard to peroxisome size, peroxisome number per cell, or activity of the major peroxisome ma-

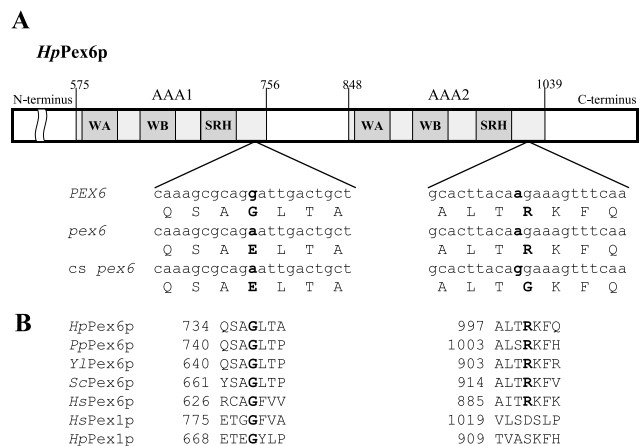


Fig. 3. Schematic representation of *HpPex6p* structure with sites of identified mutated residues. A: Two predicted nucleotide binding domains or AAA modules (in gray) are denoted as AAA1 and AAA2 with indicated numbers of flanking amino acid residues. Motifs characteristic of the AAA family of ATPases Walker A and B (WA, WB), and minimal AAA or 'second region of homology' (SRH) are shown. Mutated nucleotides and corresponding amino acid residues are shown in bold. B: Fragments of alignment of yeast and human Pex6 and Pex1 proteins comprising mutated residues (in bold). Proteins aligned were from *H. polymorpha* (Hp), *Pichia pastoris* (Pp), *Saccharomyces cerevisiae* (Sc), *Yarrowia lipolytica* (Yl), and *Homo sapiens* (Hs).

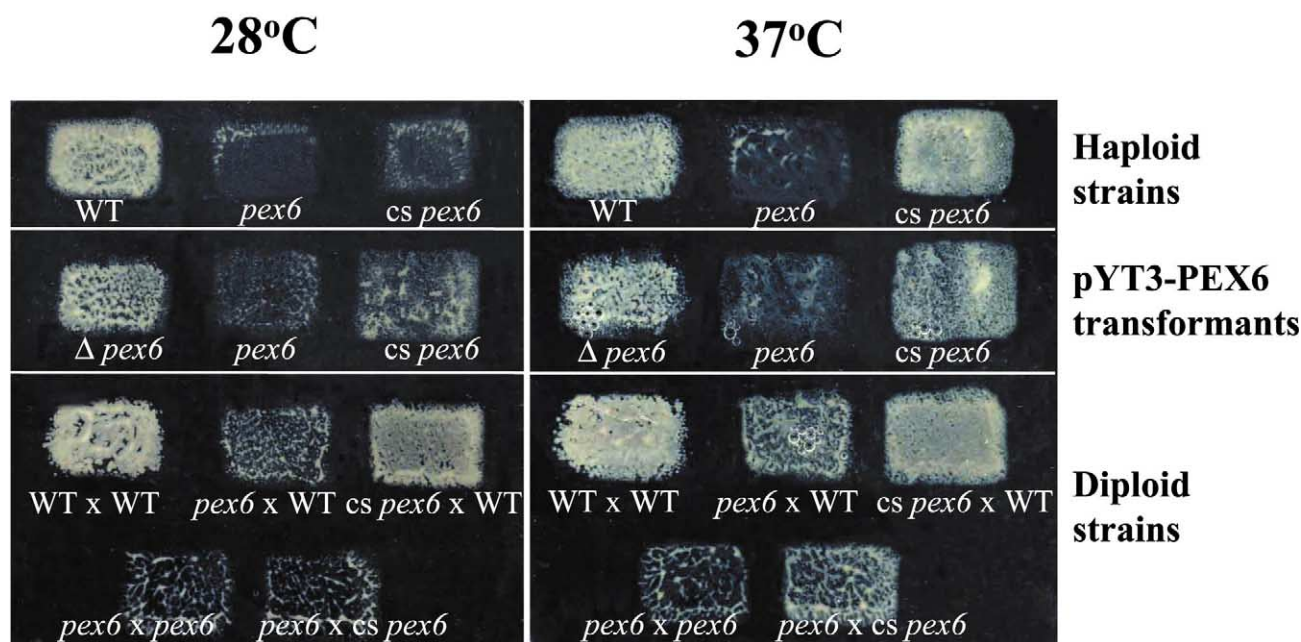


Fig. 4. Growth of *H. polymorpha* haploid strains, transformants with pYT3-PEX6 genomic library plasmid, and diploid hybrids on methanol plates at different temperatures.

trix enzyme alcohol oxidase upon methanol induction at 37°C.

At 28°C, the restrictive temperature for methylotrophic growth, morphologically altered enlarged peroxisomes were observed in most of the thin sections (Fig. 2E). Thus, a secondary mutation in *cs pex6* restored peroxisome formation at the restrictive temperature, but not organellar function necessary for methylotrophic growth.

3.3. Genetic analysis

The *cs pex6* mutant was crossed with the wild-type strain, and the resulting spore progeny of a diploid hybrid strain was analyzed for Pex[−] phenotype. Out of several thousand individual colonies only one was identified as unable to grow on methanol at 28 and 37°C. In all other cases colonies exhibited wild-type or *cs*-conditional parental phenotypes. This suggested cosegregation of an initial and a secondary mutation. Therefore, the corresponding *cs* mutation was most probably tightly linked with, or resided in, the *PEX6* locus.

3.4. Identification of the mutations

Genomic DNA fragments of 3600 bp comprising the *PEX6* gene were isolated by PCR from initial *pex6* and derivative *cs pex6* mutants as described in Section 2. Their sequencing revealed a missense mutation in the *PEX6* gene of *pex6* allele (G2210A) that leads to an amino acid substitution G737E in the deduced Pex6p. In addition to the above mutation, the second intragenic mutation for the *cs pex6* allele was identified as an A2998G transition, leading to an amino acid substitution R1000G.

The glycine G737 residue mutated in the initial *pex6* strain is conserved in yeasts and human Pex6p homologues and resides in the C-terminal part of the first AAA module of Pex6p (Fig. 3). Moreover, it is also conserved in the first AAA module of the *H. polymorpha* and human Pex1p, an interacting partner of Pex6p (Fig. 3B). This suggests the functional importance of the glycine residue at this position in Pex6p and Pex1p in lower and higher eukaryotes.

The arginine R1000 residue, mutated in *cs pex6*, resides in the second AAA module of Pex6p and is conserved only among yeast and human Pex6p homologues, but not in yeast and human Pex1p (Fig. 3). This may imply functional importance of this residue specifically for Pex6p homologues.

3.5. Complementation analysis of *cs pex6* mutant

Unexpectedly, both *pex6* and *cs pex6* strains could not be fully rescued to the wild-type phenotype when transformed with a replicative plasmid pYT3-PEX6, isolated from a *H. polymorpha* genomic library and harboring the wild-type copy of the *PEX6* gene [9] (Fig. 4). Similar growth deficiency on methanol was also exhibited by the hybrid diploid strains resulting from crosses of *pex6* or *cs pex6* mutants with the wild-type strain. The diploid carrying the mutant *pex6* and *cs pex6* alleles of the gene exhibited the cold-sensitive phenotype (Fig. 4). However, the plasmid pYT3-PEX6 fully complemented the mutant with deleted *PEX6* gene (Fig. 4). The results suggest that G737E substitution leads to a partial dominant-negative phenotype, more pronounced at 28°C.

To elucidate the phenotype, caused independently by

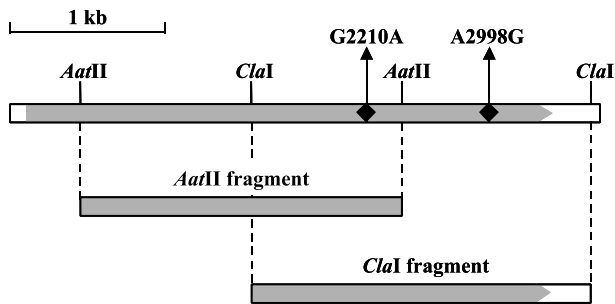


Fig. 5. Scheme showing *AatII* and *ClaI* fragments of the wild-type *PEX6* gene that were used for complementation of *pex6* and *cs pex6* mutants by homologous recombination. The *PEX6* open reading frame is shown in gray. The sites of intragenic nucleotide transitions identified in *pex6* and *cs pex6* strains are indicated.

the R1000G substitution, we transformed the *cs pex6* mutant with two different linear fragments of the wild-type *PEX6* gene isolated from the plasmid pYT3-*PEX6* by restriction enzyme digestion. The 2080-bp-long *AatII* fragment comprised a portion of the *PEX6* open reading frame with the site of the G2210A missense mutation in *pex6* (Fig. 5). The second 2199-bp-long *ClaI* fragment

harbored the C-terminal portion of the *PEX6* gene with the sites of both mutations identified in *cs pex6* (G2210A, A2998G). When transformed in *pex6* and *cs pex6* mutants by electroporation, both fragments complemented growth deficiency on methanol in these strains at 28°C, as well as at 37°C. All isolated transformants were stable with respect to methylotrophic growth, suggesting that homologous recombination occurred in the *PEX6* locus. Several corresponding transformants of the *cs pex6* mutant were analyzed for kinetics of growth in methanol medium (Fig. 6, with data for the two representative transformants provided). It was demonstrated that strains transformed with the *ClaI* fragment, able to complement both missense mutations, have wild-type kinetics of growth at 28°C and 37°C. *AatII* fragment transformants exhibited some retardation in growth at 28°C. These results suggest that complementation of the G737E substitution in *cs pex6* restores its ability to utilize methanol at the restrictive temperature. Secondly, R1000G substitution alone does not affect methylotrophic growth and peroxisome biogenesis at 37°C, and does so only to a minor extent at 28°C.

4. Discussion

In this report we describe the identification of the missense mutation in the *H. polymorpha PEX6* gene that leads to deficiency in peroxisome biogenesis and methylotrophic growth and a dominant-negative phenotype. The corresponding mutation causes an amino acid substitution in the first AAA module of the Pex6p (G737E). We also demonstrated that this mutation can be rescued to a conditional *cs* phenotype by introducing a secondary intragenic mutation in the second AAA module of Pex6p (R1000G). Both altered amino acid residues are conserved among yeast and human Pex6p homologues.

These residues are situated at C-termini of AAA modules, outside the known functionally important regions of core Walker A, Walker B, and C-terminal Sensor 1 and Sensor 2 motifs [21]. To our knowledge, mutations in the corresponding segments have not been identified or constructed for other studied AAA proteins, including human Pex1p and Pex6p homologues. C-termini in AAA modules have an α -helical composition and have been proposed to transduce changes in the nucleotide state to specific conformational changes and domain movements [22]. Interestingly, while G737E mutation abolishes Pex6p function, R1000G substitution has no significant effect when present alone. Both identified amino acid substitutions in *HpPex6p* involve a change in charge of a residue at the corresponding position (G/E, R/G). From studies of three-dimensional structures of crystallized AAA proteins and mutational analysis it is known that the two AAA modules are independent domains [23,24]. It is tempting to speculate that a complementary effect of the R1000G substitution on the G737E background is indicative of cross-

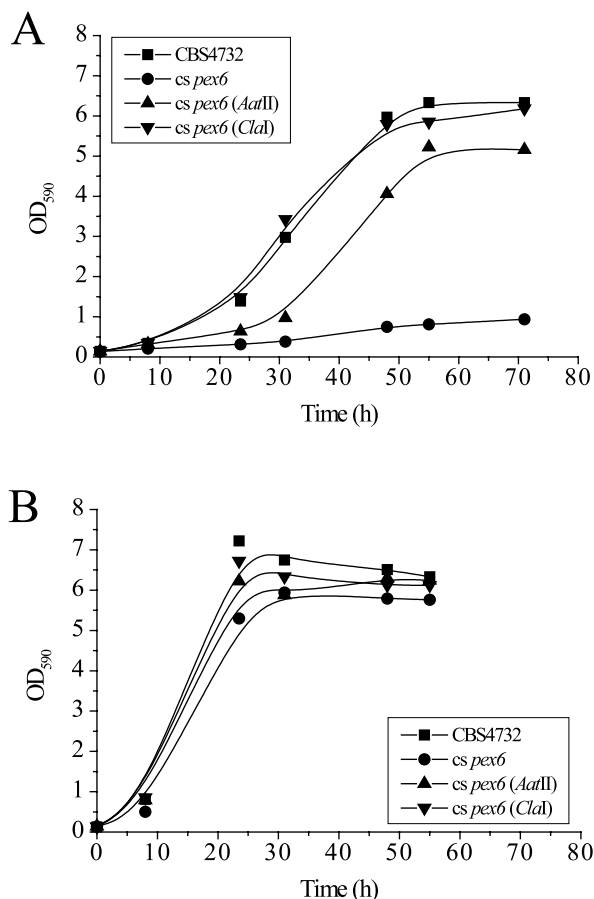


Fig. 6. Kinetics of growth of *H. polymorpha* wild-type strain, *cs pex6* mutant, and *cs pex6* transformed with linear *AatII* or *ClaI* fragments of the wild-type *PEX6* allele [*cs pex6* (*AatII*) and *cs pex6* (*ClaI*) strains] in methanol medium at (A) 28°C and (B) 37°C.

talk between the two Pex6p AAA modules, of either intra- or intermolecular character. It is also not excluded that these mutations affect Pex6p/Pex1p interaction, indispensable for peroxisome biogenesis.

The dominant character of the G737E mutation suggests that the presence of such mutated Pex6p in the cell somehow disrupts peroxisome functioning even when the wild-type copy of the protein is introduced. This *pex6* mutant will be subjected to further studies, which may provide new clues on molecular aspects of Pex6p functioning in peroxisomal import of matrix proteins.

The deficiency in methylotrophic growth in the *cs pex6* strain at the restrictive temperature is not caused by a defect in peroxisome formation, as demonstrated by the electron microscopic experiments. Restored morphologically detectable peroxisomes appear to be deficient in one of their functions at the restrictive temperature, implying a complex role of Pex6p in organelle biogenesis. Further studies will be aimed at elucidating the molecular background of the conditional phenotype in the double mutant. This strain is more sensitive to elevated concentrations of methanol than the wild-type strain, and its growth on methanol at 28°C cannot be rescued by overexpression of *HpPEX1* or *HpPEX5* genes (data not shown). These elaborations suggest that deficiency in methylotrophic growth in *cs pex6* may result from the mislocalization of peroxisomal catalase [4].

Cs pex6 mutant cells exhibited the wild-type rate of inactivation of peroxisomal alcohol oxidase when shifted from methanol to glucose medium, at both restrictive and permissive temperatures [13]. Therefore, Pex6p is not involved in autophagic peroxisome degradation, which is consistent with normal degradation of peroxisomal remnants in a mutant deleted in the *PEX6* gene [25].

Studies of the missense mutations in the *PEX6* gene may lead to a better understanding of its function in peroxisome biogenesis in yeasts, as well as in humans. Mutations in the human *PEX6* homologue (*HsPEX6*) are the second most common cause, after *HsPEX1*, of PBD (groups CG4, CG6) [8,26]. It has been demonstrated that nonsense mutations, deletions and those mutations affecting splicing severely affect *HsPex6p* function and lead to the lethal Zellweger syndrome [27,28]. Missense mutations in *HsPEX6*, as well as in *HsPEX1*, cause milder forms of PBD such as neonatal adrenoleukodystrophy and infantile Refsum disease [27,29,30]. In many cases, such mutations exhibit a temperature-sensitive phenotype, and peroxisome biogenesis can be restored in patients' fibroblasts at lowered temperatures [28,30,31]. In this respect, it should be noted that most of the missense mutations in *HsPEX6* and *HsPEX1* actually disrupt interaction of their protein products [8,32]. Studies have been initiated to develop pharmacological approaches for treating patients with such milder forms of PBD [33]. Therefore, yeast mutants, including those with introduced known human mutations, can serve as a convenient model system for study-

ing molecular mechanisms of functioning of Pex6p and other *PEX* products in peroxisome biogenesis.

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